JOURNAL OF VIROLOGY, Apr. 2010, p. 3690–3694 Vol. 84, No. 7
0022-538X/10/$12.00 doi:10.1128/JVI.02715-09

NOTES

Isolation of an Infectious Endogenous Retrovirus in a Proportion of Live Attenuated Vaccines for Pets▽†

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Received 24 December 2009/Accepted 19 January 2010

The genomes of all animal species are colonized by endogenous retroviruses (ERVs). Although most ERVs have accumulated defects that render them incapable of replication, fully infectious ERVs have been identified in various mammals. In this study, we isolated a feline infectious ERV (RD-114) in a proportion of live attenuated vaccines for pets. Isolation of RD-114 was made in two independent laboratories using different detection strategies and using vaccines for both cats and dogs commercially available in Japan or the United Kingdom. This study shows that the methods currently employed to screen veterinary vaccines for retroviruses should be reevaluated.

During evolution, the genomes of all animal species have been colonized by endogenous retroviruses (ERVs). ERVs are derived from the integration of the retrovirus genome ("provirus") into the host germ line and are transmitted vertically between generations like any other Mendelian gene (3). Most ERVs have accumulated mutations and/or deletions that render them unable to complete their replication cycle. However, there are several examples of ERVs that have been shown to be fully infectious. These replication-competent ERVs, especially those that have coevolved with their host for long evolutionary periods, can be considered in many ways to be in equilibrium with their host species, which have adopted a variety of strategies to suppress and control viral expression and/or replication.

Regardless of their replication potential, ERVs in general can be considered nonpathogenic for their host, otherwise they would have been counterselected during evolution (1, 11). However, transmission of infectious ERVs to an animal species different from the one in which they originally integrated ("cross-species transmission") could have unpredictable outcomes. For this reason, the potential transmission of pig ERVs to humans is one of the blocks hampering xenotransplantation (26).

Here, we wanted to evaluate the possibility that live attenuated vaccines could contain replication-competent ERVs and act as a potential source of retroviral cross-species transmission. We investigated commercially available vaccine preparations for cats and dogs. The cat genome contains an infectious ERV known as RD-114, a member of the *Gammaretrovirus* genus, to which other mammalian oncogenic viruses such as feline leukemia virus (FeLV) and murine leukemia virus belong (6, 14, 17, 23). Some feline cell lines such as CRFK (Crandell-Rees feline kidney) commonly used to grow feline and canine viruses express variable amounts of RD-114 (2). We attempted to isolate RD-114 from vaccines commercially available in different continents. These vaccines are routinely used to prevent common infections in cats and dogs caused by viruses such as feline herpesvirus, feline calicivirus, feline panleukopenia virus, canine adenovirus, canine distemper virus, canine parvovirus, canine coronavirus, and canine parainfluenza virus (15). For this study, each vaccine sampled was assigned an anonymized code (e.g., J-Aa1, UK-Aa4 etc.). The first letter before the dash indicates the country where the vaccine was acquired (i.e., "J" for "Japan" and "UK" for the United Kingdom). The capital letter after the dash indicates the manufacturer. The lowercase letter indicates the specific type of vaccine, while numbers are used to differentiate between different batch numbers. Thus, vaccines J-Aa1 and UK-Aa4 are two different batches of the same vaccine acquired from either Japan (J-Aa1) or the United Kingdom (UK-Aa4). All of the data obtained in this study are summarized in Table 1.

Initially, 15 samples of 11 vaccines acquired from Japan (from 7 different manufacturers) were tested for the presence of RD-114 using a LacZ marker rescue assay as previously described (19) (Fig. 1A to F). Briefly, vaccines (one vial per each vaccine) were passaged for 4 weeks (dog vaccines) or 2

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▽ Published ahead of print on 27 January 2010.

† The authors have paid a fee to allow immediate free access to this article.

TABLE 1. Detection of RD-114 in commercially available vaccines for dogs and cats in this study

Vaccine code[a]	Target species[b]	No. of positive results/no. of vials tested by[c]:	
		LacZ or Western blotting LacZ[d]	PCR or RT activity-PCR[e]
J-Aa1	**Cats**	**3/6**	**2/2**
J-Aa2	**Cats**	**1/1**	**1/1**
J-Aa3	**Cats**	**0/2**	**0/2**
J-Bc1	Cats	0/3	0/3
J-Bc2	Cats	0/3	0/3
J-Cd1	Dogs	2/2	2/2
J-Co1	Dogs	1/1	1/1
J-Co2	Dogs	1/1	1/1
J-De1	**Dogs**	**1/1**	**1/1**
J-Ef1	Dogs	0/1	0/1
J-Gk1	Dogs	0/1	0/1
J-Ab1	Dogs	0/1	0/1
J-Gl1	Dogs	0/1	0/1
J-Em1	Cats	0/1	0/1
J-Hn1	Cats	0/3	0/1
		Western blotting[f]	RT[g]
UK-Aa4	**Cats**	**3/4**	**2/3**
UK-Aa5	**Cats**	**0/2**	**0/2**
UK-Bi1	Dogs	0/2	0/2
UK-De2	**Dogs**	**2/2**	**2/2**
UK-De3	**Dogs**	**1/4**	**1/3**
UK-Eg1	Dogs	0/1	nt
UK-Gk2	Dogs	0/2	0/2
UK-Eh1	Dogs	0/2	0/2
TE671	–	0/4	0/2
RD114 (+)	–	4/4	2/2

[a] Codes used to anonymize the vaccines used. The first letter before the dash indicates the country where the vaccine was acquired ("J" for Japan or "UK" for the United Kingdom). The capital letter after the dash indicates the manufacturer. The letter in lowercase indicates the specific type of vaccine, while numbers are used to differentiate between different batches. Boldface entries for vaccines J-Aa, J-De, UK-Aa, and UK-De highlight the same vaccine brands (but different batches) purchased in either Japan or the United Kingdom. RD-114(+) represents the positive control obtained from supernatants of feline FER cells (2). TE671 are the mock-infected cells used as a negative control.

[b] Animal species to which each specific vaccine is targeted.

[c] For each vaccine, values refer to the number of samples that tested positive for RD-114 compared to the total number of vials tested.

[d] Data relative to the LacZ marker rescue assay using one vial of the various vaccines in each assay.

[e] Results of the RD-114 *env* PCR on TE671 cells exposed to the indicated vaccines.

[f] Data relative to the Western blotting analysis of supernatants of TE671 cells exposed to the indicated vaccines (two vials for each assay).

[g] Vaccines were tested for the presence of reverse transcriptase activity using C-type RT activity kit (Cavidi) as described by the manufacturer.

weeks (cat vaccines) into TE671(LacZ) cells, which are derived from the human rhabdomyosarcoma cell line TE671 transduced with a gammaretroviral vector expressing the *lacZ* gene. Supernatants from the vaccine-inoculated TE671(LacZ) cells were collected and used to infect naïve TE671 cells. The LacZ marker rescue assay showed the presence of a replication-competent gammaretrovirus in 6 of the 15 samples tested (Fig. 1A and C). On the other hand, the same vaccine preparations were not able to infect TE671-RD, a cell line chronically infected with RD-114 (Fig. 1B and D). TE671-RD cells were infectible by a retroviral vector pseudotyped with the feline leukemia virus subgroup B (FeLV-B) Env, demonstrating that these cells can be infected by a retrovirus that uses a different receptor from RD-114 (Fig. 1E and F). We estimated the infectious titers of RD-114 in some of the vaccines tested above by an endpoint dilution assay. Briefly, TE671(LacZ) cells were exposed with serial dilutions of vaccines J-Aa2, J-Cd1, J-Co2, and J-De1. Viral titers were calculated using the formula of Reed and Munch (16). Titers were as low as 1.8 50% tissue culture infective doses ($TCID_{50}$)/vial in vaccines J-Aa2 and J-De1. Higher titers were reached in vaccines J-Co2 (1,000 $TCID_{50}$) and J-Cd1 (1,800 $TCID_{50}$). Note that the titers indicated above are an estimate that may contain a certain degree of variability because most of the dog vaccines tested contains viruses that are cytopathic in TE671 cells. Thus, fresh TE671(LacZ) cells were continuously added to the cultures exposed to the pet vaccines in order to allow sufficient time for RD-114 replication.

A single-step PCR assay employing RD-114-specific primers was also employed on genomic DNA (200 ng/reaction) extracted from TE671(LacZ) infected with the vaccines described above (Fig. 1G). The primers employed were designed to amplify the RD-114 *env* region using the following oligonucleotide primers: 5′-CCCTCGATACTAAGAGAGTG-3′ and 5′-ACTTCAGCTAACGAGTCTAC-3′. We were able to amplify RD-114 *env* sequences in all samples that tested positive in the LacZ marker rescue assay, confirming the presence of RD-114. On the other hand, vaccines that tested negative in the LacZ marker rescue assay were also found to be PCR negative for RD-114. Electron microscopy of human TE671(LacZ) cells infected with vaccine J-Aa1 and passaged for 4 weeks showed the presence of viral particles with retroviral morphology (Fig. 1H).

We then isolated RD-114 in another laboratory, using pet vaccines commercially available in the United Kingdom and using a different strategy. Eight different samples (two vials of each vaccine in each experiment), representing 6 vaccines from 6 different manufacturers, were passaged for 3 to 6 weeks on TE671 cells, and culture fluids were tested for the presence of RD-114. Two of the 6 vaccines tested were found to contain RD-114, as assessed by Western blotting employing an antiserum raised against the RD-114 major capsid protein (CA) and by a reverse transcriptase (RT) assay (Fig. 2 and Table 1). In general, cells exposed to vaccines containing RD-114 had to be passaged for 3 weeks before RT activity could be detectable (Fig. 2C). Interestingly, the same vaccine brands were found to contain RD-114 in the United Kingdom (UK-Aa and UK-De) and in Japan (JAa and J-De). Note that in some of the vaccine brands we were not able to detect consistently RD-114 in all of the batches tested (J-Aa and UK-Aa) or in all of the vials from the "contaminated" vaccine batches (i.e., J-Aa1, UK-De3, and UK-Aa4) (Table 1). As mentioned above, this is likely due to the small amount of RD-114 present in the vaccine seeds and/or to inherent variability of the tests due to the presence of viruses in the vaccine formulations that are cytopathic to TE671 cells. A small number of experiments were performed also on human 293T cells. We were able to isolate RD-114 in 293T from vaccine UK-De3. However, these cells were more susceptible than TE671 cells to the cytopathic effect induced by viruses contained in the vaccine preparations, and therefore we used the latter for the complete experimental set relative to the United Kingdom samples. Overall, it is possible that our data underrepresent the number of vaccines from which RD-114 can be isolated.

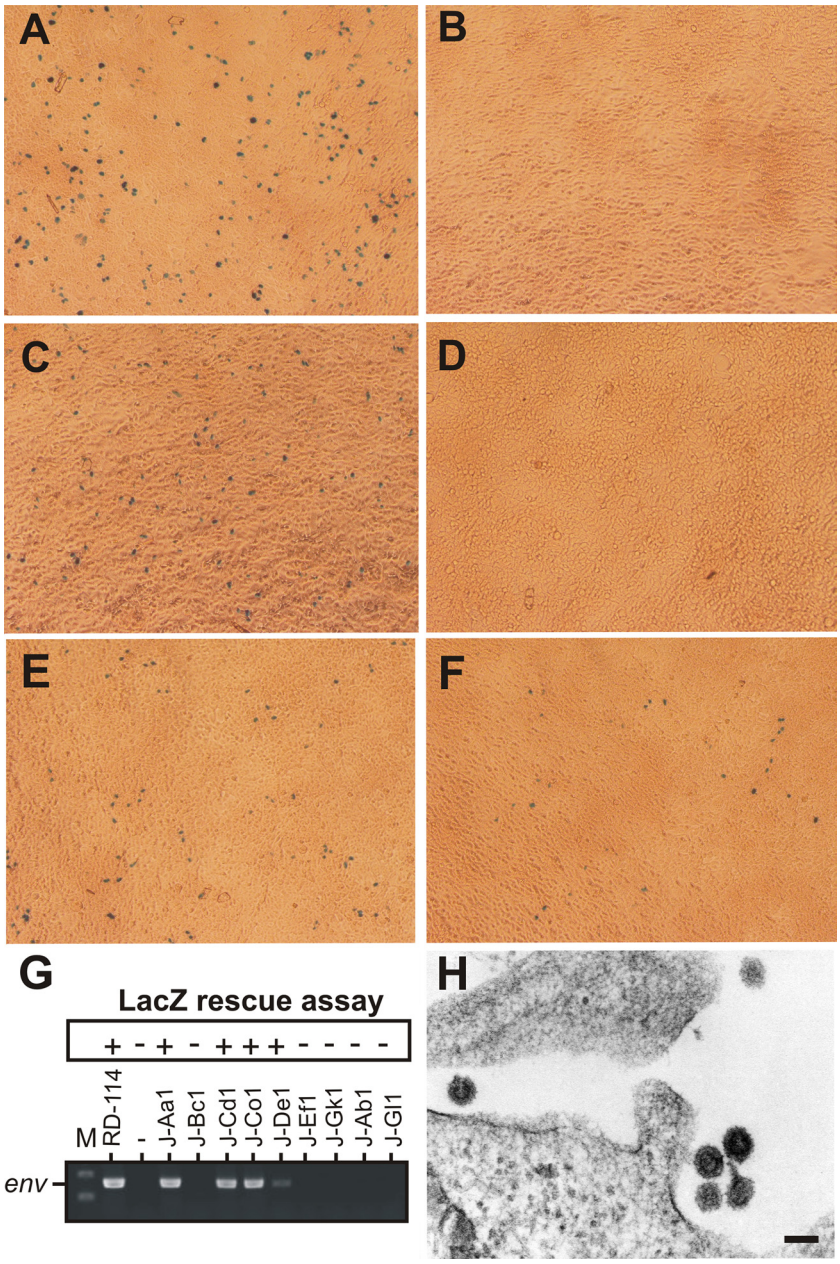

FIG. 1. Detection of RD-114 in pet vaccines. A total of 14 vaccine brands acquired from 7 different vaccine manufacturers were tested (also see Table 1 for a complete summary of the data obtained). Four of the 14 vaccines tested in both Japan and the United Kingdom showed evidence of RD-114 in at least one of their batches. Two of the vaccines (J-Cd and J-Co) from which RD-114 was isolated were commercially available only in Japan, while the remaining two were present in both markets (J-Aa, J-De, UK-Aa, and UK-De). Consequently, the presence of RD-114 was revealed in two different laboratories in the same vaccines (J-Aa/UK-Aa and J-De/UK-De), using independent samples, reagents, and detection strategies. (A to E) LacZ marker rescue assays were performed as already described (19). Panels A and C show representative examples of TE671 cells exposed to a vial containing vaccines J-De1 and J-Co1 respectively. The same vaccine preparations were not able to infect TE671-RD, a cell line chronically infected with RD-114, as shown in panels B and D. As a control, both TE671 and TE671-RD were infected by a murine leukemia virus-based vector pseudotyped with the FeLV-B Env (E and F). (G) RD-114 provirus was amplified from genomic DNA (200 ng/reaction) extracted from TE671(LacZ) cells exposed to the indicated vaccines. Reactions were carried out using the primers indicated in the text in a 25-μl standard PCR. The PCR cycles employed were 94°C for 10 min and 30 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 90 s, with a final extension of 72°C for 10 min. Specific PCR products were obtained only from those samples for which there was a positive result in the LacZ assay, as indicated schematically in the figure. PCR products were sequenced and confirmed to represent RD-114 *env*. (H) Visualization of retroviral particles by electron microscopy. TE671(LacZ) cells infected with vaccine J-Aa1 were passaged for 4 weeks. Cells were then fixed, dehydrated, embedded, and sectioned for electron microscopy by using standard methods. The microphotograph shows mature viral particles with a retroviral morphology in the vicinity of the cell membrane (bar, 100 μm).

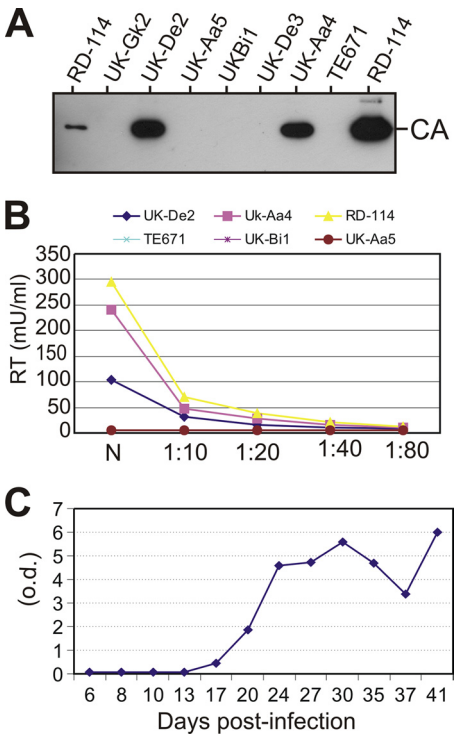

FIG. 2. Detection of RD-114 by Western blotting and reverse transcriptase (RT) assay. (A) Vaccines obtained from the United Kingdom were passaged in TE671 cells for 3 to 6 weeks. The samples represented in the figure were taken at the end of the experiment at 6 weeks after the initial exposure of TE671 cells to the indicated vaccines. TE671 cells were exposed to two vials of vaccines in each experiment. Approximately 10 ml of culture supernatants was collected, filtered through a 0.45-μm filter, and ultracentrifuged at 100,000*g* for 1 h. Virus pellets were resuspended in 50 μl Tris-EDTA (TE) buffer. Twelve microliters was then analyzed by Western blotting using a goat antiserum against the RD-114 major capsid protein (serum 72-S-781 from the U.S. National Cancer Institute). Note the presence of RD-114 CA in supernatants of cells infected with vaccines UK-De2 and UK-Aa4. As a positive control (lanes labeled "RD-114"), we utilized supernatants obtained from the feline cell line FER that expresses RD-114 (2). The specificity of the antibody used was also tested by transfecting 293T cells transfected with an infectious molecular clone of RD-114 as previously described (13). (B and C) RT assays were performed using the C-type RT activity kit (Cavidi) as recommended by the manufacturer. Panel B show representative data from some of the vaccines tested. Values are normalized against a Moloney murine leukemia virus recombinant RT standard and expressed in milliunits (mU) per ml. Note that culture supernatants were taken either undiluted ("neat") or diluted as indicated in the graph. All samples that contained detectable RD-114 by Western blotting also had detectable RT activity. Panel C shows RT assays of supernatants of TE671 cells infected with vaccine UK-Aa4 and collected at different times postinfection. Note that RT activity is above the detection limit only after 3 weeks postinfection. An aliquot of the same sample collected at day 41 postinfection is the Uk-Aa4 sample shown also in panel B. o.d., optical density.

Collectively, our data show unequivocally that RD-114 is present in live attenuated vaccines commonly used in dogs and cats from different continents and produced by three different manufacturers. Future studies will be necessary to determine whether RD-114 has any negative impact in cats or dogs. The risks posed by a low-level exposure to RD-114 for pets are likely extremely small. ERVs are in general nonpathogenic for their host species, and RD-114 replication has been found to be restricted in some cat cell lines (4, 5, 12). However, cat cell lines fully susceptible to RD-114 replication have also been described (7). Thus, one would expect that exposure to RD-114 will be of little consequence to cats, given that RD-114 is an ERV of this species. However, it is impossible to rule out any consequences at all in the pet population as a whole. Infectious ERVs have the same biological properties and pathogenic potential of exogenous horizontally transmitted retroviruses, once the coevolutionary mechanisms that have shaped the interaction with their natural hosts cease to exist. In this regard, the large-scale exposure to RD-114, particularly of the dog population, may have effects that are impossible to predict even if successful RD-114 transmission was an extremely rare event. Millions of puppies are vaccinated annually worldwide, and they may be more susceptible to RD-114 infection than cats as the dog genome does not harbor RD-114. Also wild cats do not harbor RD-114, and they are regularly vaccinated in zoos with the same vaccines used for pets. These vaccines have been used extensively for many years without major acute effects on vaccinated animals, but retroviruses rarely induce acute diseases. Therefore, it is impossible to rule out chronic effects, especially as we were able to grow RD-114 very efficiently in dog cell lines (data not shown), confirming older published studies (18).

To our knowledge, this is the first identification of an infectious ERV in commercially available vaccines. In the 1960s, it was shown that yellow fever live attenuated vaccines prepared in chicken embryo fibroblasts were contaminated with avian leukosis virus (ALV). However, no increased risk for cancer was shown in vaccinated individuals, and this was expected considering that ALV does not grow efficiently in mammalian cells (8, 24, 25). Avian endogenous retrovirus genomes and RT activity have been detected more recently in yellow fever, measles, and mumps vaccines by RT and RT-PCR assays (9, 21), but no evidence of infectious viruses was presented in these studies. As expected, avian ERVs were not detected in the vaccine recipients.

A recently identified novel human retrovirus (xenotropic murine leukemia virus-related retrovirus [XMRV]) has been found in some forms of prostate cancers and chronic fatigue syndrome in humans (13, 20, 22), although causal association has not been proven yet. XMRV is almost undistinguishable from an ERV present in mice, and it will be important to investigate how this virus passed into the human population, regardless of its pathogenic potential. Interestingly, the current methods used for screening human vaccines for retroviral contaminants include extremely sensitive PCR-based RT assays (not required for veterinary vaccines) that are much more sensitive than conventional RT assays. Thus, contamination of human vaccines with XMRV would not pass undetected with the currently available technology, although this may not be necessarily true for vaccines produced in previous decades.

Finally, although the risks posed by RD-114 are seemingly small, it would be appropriate to produce live attenuated vaccines in cells that do not express this endogenous retrovirus. To this end, cells of dog origin may be better suited to produce pet vaccines than cat cell lines, although not all cat cell lines express RD-114 (2, 10). Contamination of subunit or inactivated

vaccines by infectious agents in general (including ERVs) is obviously less of a concern.

In conclusion, our study suggests that the presence of infectious ERVs should be taken into consideration when assessing the purity of live attenuated veterinary vaccines. The methods currently employed to screen veterinary vaccines for retroviruses need to be reassessed.

We are grateful to Roger Reeves for providing an RD-114 infectious molecular clone and Hans Lutz for antibodies against the RD-114 major capsid protein. We are grateful to Claudia Seewald, Margaret Hosie, Brian Willett, Os Jarrett, Fred Arnaud, Pablo Murcia, Mark Jackson, and Mariana Varela for useful suggestions.

This work was funded by the Wellcome Trust and by the Bio-Oriented Technology Research Advancement Institution in Japan.

The European Medicines Agency and vaccine manufacturers are aware of the possible presence of RD-114 in some vaccines for veterinary use since 2008. The Agency has agreed with vaccine manufacturers to study methods to allow a full assessment of the potential significance of the findings.

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